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1 **Functional polymers alterations by phylloplane bacteria isolated from crops**

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9 **Abstract**

10 The growing uses of functional polymers in different domains and the well-established remanence of
11 synthetic polymers underline the necessity to study their microbial biodegradation. A phylloplane
12 bacterial collection was screened for its potential to overcome documented factors limiting polymer
13 biodegradation, i.e. low cleavable long chain linear polymers and low bioavailable hydrophobic
14 compounds. Acrylate-based polymers, namely poly(2-hydroxyethyl methacrylate) (pHEMA), poly(N-
15 isopropylacrylamide) (pNIPAM), poly(acrylamide) (pAM) and poly(acrylic acid sodium salt) (pAS), and
16 a hydrophobic crosslinker Epoxidized Soybean Oil Acrylate (ESOA) were used as selective substrates.
17 OD monitoring of growth with these molecules as sole carbon sources were correlated with
18 substrates alterations determined by ¹H NMR, GPC and FTIR. Selected strains belong to Bacillales
19 Family XII. Incertae Sedis, Bacillaceae, Nocardiaceae, Micrococcaceae, Enterobacteriaceae and
20 Pseudomonadaceae. *Bacillus* species mainly developed with ESOA. *Exiguobacterium sibiricum* strains
21 could grow with pHEMA and pNIPAM leading to polymer molecular weight decreases. *Arthrobacter*
22 *koreensis* showed the highest alteration capacities of pHEMA with molecular weight decreases from
23 22,000 to 3,148 g/mol and the production of presumed aromatics and carbon chains.

24 **Key words**

25 Biodegradation; Hydrophobic monomer; Long chain backbones; Natural biodiversity; Crops
26 phyllosphere; Acrylate derivatives

27 1. Introduction

28 Numerous studies have recently focused on the environmental fate and biodegradation of
29 packaging plastics due to their high stability and their resistance to environmental stresses
30 (Restrepo-Flórez *et al.*, 2014). Nevertheless, other classes of polymeric materials such as engineering
31 and high-performance plastics did not draw as much attention despite several characteristics
32 identified as biodegradation or bioavailability key limiting factors, e.g. long backbones or
33 hydrophobicity (Leja and Lewandowicz, 2009).

34 Polyacrylate based polymers and notably poly(2-hydroxyethyl methacrylate) (pHEMA),
35 poly(*N*-isopropylacrylamide) (pNIPAM), poly(acrylamide) (pAM) and poly(acrylic acid sodium salt)
36 (pAS) have been widely used in biotechnological and industrial applications. pHEMA, pNIPAM and
37 pAS have found applications in biomedical devices (Huang *et al.*, 2013; García-Millán *et al.*, 2015;
38 Huber *et al.*, 2016; Taddei *et al.*, 2017), cell culture materials (Rzaev *et al.*, 2007) or drug delivery
39 systems (Trigo *et al.*, 1994; Liu *et al.*, 2016). pNIPAM, pAM and pAS are used in water treatment as
40 flocculants or for their sorbent properties (Kenawy *et al.*, 2013; Lee *et al.*, 2014; Nga *et al.*, 2018;
41 Wong *et al.*; 2006). pAS has been employed as water sorbent in agricultural soils (Wenhua *et al.*,
42 2008).

43 The areas of use of these functional polymers expose them to shear, oxidative, thermal or
44 biological stresses that may cause wear and a partial or total release in the environment either as
45 dust or water effluents. Besides, direct amendments to prevent post-fire runoff, soil erosion (Prats *et al.*,
46 2014) or heavy metal pollutions as well as waste disposal are sources of soil contaminations
47 (Santos *et al.*, 2013). However, the end of life and environmental fate of functional polymers are still
48 not well determined. Indeed, as biomedical device component, pHEMA biodegradation was mainly
49 studied in mammals rather than in microorganisms. A previous study of our laboratories reports
50 the isolation of bacterial strains able to grow with pHEMA as sole carbon source from an oil-
51 contaminated soil, but polymer chemical alterations were not characterized (Zhao, 2015). Similarly,
52 few studies extensively investigated pNIPAM toxicity and biodegradation, although the monomer is
53 reported as a toxic compound (U.S. EPA DSSTox Database, Substance ID: DTXSID0033754). *Pantoea*
54 (*Enterobacter*) *agglomerans*, *Azomonas macrocytogenes* (Nakamiya and Kinoshita, 1995), *Bacillus*
55 *cereus* (Wen *et al.*, 2010), *Bacillus sphaericus* and *Acinetobacter sp.* (Matsuoka *et al.*, 2002) strains
56 were reported able to grow with pAM as sole carbon source. To our knowledge, *Arthrobacter sp.* NO-
57 18 (Hayashi *et al.*, 1993) is the only identified bacteria reported to catabolize pAS as pure culture.

58 Vegetable oils have been widely investigated as a valuable alternative to hydrocarbons for
59 the development of new plastic materials (Islam *et al.*, 2014; Samarth and Mahanwar, 2015; Hatti-
60 Kaul *et al.*, 2019). In particular, the modification of vegetable triglycerides by epoxidation and the
61 ring-opening with acrylic acid derivatives formed new bio-based monomers such as Epoxidized
62 Soybean Oil Acrylate (ESOA). ESOA can be used for the preparation of polymers with high impact
63 resistance, low electrical properties and high hydrophobicity (Zhang *et al.*, 2017). However, bio-
64 sourcing does not always imply a good biodegradability and the environmental fate of this new
65 generation of oily substrates has mostly to be investigated.

66 Phyllosphere constitutes a naturally hydrocarbon rich compartment, mainly composed of
67 plant waxes (Riederer and Müller, 2006) consisting in acids, alcohols, esters, aldehydes, ketones and
68 alkanes with very long carbon chains (Kunst and Samuels, 2003; Buschhaus *et al.*, 2007; Buschhaus
69 and Jetter, 2012) and some cyclic terpenoids in the intracuticular wax layer. This specific
70 environment is likely to favor microbial adaptations enabling enhanced access toward hydrophobic
71 substrates and metabolization of complex compounds. Incidentally, several efficient hydrocarbon-
72 degrading bacteria have been isolated from the phyllosphere such as *Acinetobacter*, *Pseudomonas*,
73 *Mycobacterium*, *Arthrobacter*, *Bacillus*, *Rhodococcus* (Yutthammo *et al.*, 2010, Al-Awadhi, 2012).
74 Besides, phyllosphere was not reported to increase human pathogens proportion within microbial
75 communities in contrast to plastics impacted environments (Jacquin *et al.*, 2019; Puglisi *et al.*, 2019).

76 In this study, we hypothesize that crops phyllosphere natural biodiversity includes bacteria
77 capable of altering functional polymers through their adaptation to low cleavable long linear chains
78 and low bioavailable substrates.

79 2. Materials and Methods

80 2.1. Leaves sampling

81 Entire leaves without any visible lesions were collected from crop fields in the Hauts-de-
82 France region (France) using single-use clean plastic bags and laboratory gloves and were kept at 4°C
83 for 1 month until the isolation step. Four crops were selected and identified through the information
84 about the fields and their morphological properties (Bonnier and De Layens, 1986): cabbage (*Brassica*
85 *oleracea* L.), corn (*Zea mays* L.), rape (*Brassica napus* L.) and sugar beets (*Beta vulgaris* L.).

86 2.2. Suspension dilution isolation technique

87 50 disks of 1 cm² were cut out from the leaves limb of each plant variety with a sterile round
88 cutter. Disks were soaked in 50 ml of sterile water in a 100 ml flask (adapted from Dickinson *et al.*,
89 1975). The flask was agitated 1h at room temperature on an orbital shaker at 150 rpm and then
90 sonicated 1 min at 47 Hz. 100 µl of 10-fold serial diluted suspensions (10⁻¹, 10⁻² and 10⁻³) were plated
91 on Tryptic Soy Agar (TSA) containing 5 mg/L of cycloheximide to inhibit fungal development. The
92 Petri dishes were incubated at 35°C over a week. Single colonies were isolated every day on fresh
93 TSA medium by the streaking method and kept at 4°C after that colonies developed.

94 2.3. Foliar imprints isolation technique

95 For each plant variety, a disk of 1 cm² was cut into a leaf with a sterile round cutter. The
96 upper face was pressed directly on a TSA plate with sterile tweezers. Successive imprints were done
97 on a same Petri dish (adapted from Dickinson *et al.*, 1975). The same protocol was applied to the
98 lower face of the leaf. This protocol was repeated three times per plant. Petri dishes were incubated
99 over a week at 35°C. Single colonies were daily sampled from the imprints, isolated on TSA medium
100 by the streaking method and conserved at 4°C.

101 2.4. Sequencing and Phylogenetic Analysis

102 DNA was extracted from isolated bacteria freshly cultured on LB medium according to
103 Serghini *et al.* (1989) and quantified by OD at 260 nm. 250 ng of each DNA sample was submitted to
104 hot-start amplification (40 cycles: 2 min 94°C, 1min 55°C, 1 min 72°C; final elongation: 7 min 72°C) in
105 a Perkin-Elmer/Cetus 480 DNA Thermal Cycler. The 16S 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and
106 534-R (5'-ATTACCGCGGCTGCTGG- 3') PCR primers encompassing V1 to V3 hypervariable regions of
107 16S rDNA (Lane, 1991; Turner *et al.*, 1999) were obtained from Eurofins Genomics (Ebersberg,
108 Germany). The other PCR components were purchased from New England Biolabs France (Evry,
109 France) (Muyzer *et al.*, 1993). PCR amplification products were verified on a 1% agarose gel. Sanger
110 sequencing data were generated by Eurofins Genomics (Ebersberg, Germany) and were compared to
111 the Ribosomal Database Project (RDP release 11, <http://rdp.cme.msu.edu>) and GenBank at NCBI
112 (<https://blast.ncbi.nlm.nih.gov>) with Basic Local Alignment Search Tool (BLAST) (D'Aquila *et al.*,
113 1991), allowing strains identification. The sequences have been registered in Genbank under
114 accession numbers MT159394-MT159410.

115 2.5. Microplate OD variation measurement screening method

116 10 ml of TSB (Tryptic Soy Broth) in 50 ml conical bottom tubes were inoculated with colonies
117 of each isolate and incubated 24h at 30°C on a rotary shaker (180 rpm). After centrifugation (3000
118 rpm, 10 min), pellets were washed twice by manual shaking with Mineral Medium (MM), (250 mg of
119 KCl, 6.464 g of NaH₂PO₄, 2H₂O, 10.408 mg of Na₂HPO₄, 2H₂O, 244 mg of MgSO₄, 7H₂O, 1 g of NO₃NH₄,
120 10 µg of ZnSO₄, 7H₂O, 1 µg of MnCl₂, 4H₂O, 10 µg of FeSO₄, 7H₂O, 5 µg of CuSO₄, 1 µg of CaCl₂, 2 µg of
121 MoO₃ per liter, pH 7) for 10 minutes to eliminate nutrients, particularly carbon sources remaining
122 from TSB. Final pellets were suspended in MM by manual shaking and successive pipetting until
123 disappearing of aggregates and diluted to reach an absorbance of 0.4 at 600 nm. 10 µl of this
124 suspension were inoculated to microplates (96 well NuncTM edge 2.0 micro-plates, ThermoFisher
125 Scientific) already containing 190 µl of mineral medium supplemented with the different carbon
126 sources (carbon equivalent concentration: 2 g/L, table 1). The selective substrates pHEMA (20,000
127 g/mol), pNIPAM carboxylic acid terminated (5,000 g/mol), pAM (40,000 g/mol), pAS (5,100 g/mol, 8-
128 14 % water) and ESOA (purity ≥ 99 %, contains 4,000 ppm monomethyl ether hydroquinone as
129 inhibitor) were purchased from Merck (France). ESOA was dispersed in the mineral medium by
130 sonication, 2 minutes, 40 kHz (Branson Branson[®] CPXH 5800, Emerson). Negative controls

131 (inoculated MM without carbon source) and positive controls (inoculated MM containing 5 g/L of
132 glucose) were included and culture conditions were conducted in triplicates. Triplicates of non-
133 inoculated MM with glucose were also used as blank to check the absence of contamination.
134 Micro-cultures were incubated at 30°C under constant agitation and bacterial growth was estimated
135 after 7 days of incubation measuring absorbance at 600 nm (Multiskan GO microplate reader, SkanIt
136 program, ThermoFisher Scientific). Isolates showing the highest absorbance variations with the best
137 diversity of substrates were selected for further studies. Results of the selected strains were
138 processed through Principal Component Analysis with a Pearson correlation model using the XLSTAT
139 software.

140 2.6. Macro-cultures and growth kinetic monitoring

141 OD_{600 nm} evolution kinetics were performed on the selected isolates using macro-cultures.
142 Inocula were prepared and calibrated as described in section 2.5. 5 ml of mineral medium containing
143 the selected substrates (table 1) were distributed in 15 ml bottom tubes, inoculated with 250 µl of
144 adjusted bacterial suspension. 200 µl of these macro-cultures were immediately transferred in a 96-
145 well microplate in triplicate and incubated 4 days at 30°C under constant agitation in order to follow
146 the bacterial growth kinetics in parallel to the macro-cultures incubation. Growth kinetics were
147 followed at 600 nm (Day 1: every 15 minutes, Day 2 : every 30 minutes, Day 3 and 4 : every hour). In
148 parallel, the leftover macro-cultures were incubated at 30°C under constant agitation (180 rpm).
149 After 4 days, macro-cultures were centrifuged 10 min at 3000 rpm, supernatants were kept for
150 analytical measurements and pellets were freeze dried to determine the dry biomass weights. To
151 assess abiotic polymer modifications and losses, polymers in MM were incubated in the same
152 conditions.

153 Growth kinetics of cultures were characterized determining maximal ΔOD_{600nm} over 7 days and
154 exponential growth rate μ , defined by the equation below

$$155 \mu = \frac{\ln OD_{600nm ty} - \ln OD_{600nm tx}}{\ln t_y - \ln t_x}$$

156 where t_x and t_y are exponential period boundaries. Results are presented as averages over triplicates
157 with the corresponding standard deviations as error bars.

158 Growth kinetics of the strains studied for polymers alteration are presented as curves of the average
159 OD_{600nm} of triplicates according to time with corresponding standard deviations as error bars.

160 2.7. Polymer structure analyses

161 ATR-FTIR spectra (4000-600 cm⁻¹, 200 scans, 4 cm⁻¹ resolution) were measured on cultures
162 and controls supernatants using a Thermo Nicolet 6700 spectrophotometer (ThermoFisher scientific)
163 equipped with a ZnSe ATR (attenuated total reflectance) system and MCT-B detector.

164 ¹H NMR was performed on all the polymers and culture supernatants. NMR spectra were
165 recorded on Bruker Avance 400 MHz spectrometer using a direct BBFO probe-head. 600 µl of the
166 culture supernatants were freeze dried and the dry samples were solubilized in 600 µl D₂O for
167 pHEMA, pNIPAM and CDCl₃ for ESOA. Pyridine was used as an internal standard at a concentration of
168 10 µM.

169 The polymers molecular weight was determined by gel permeation chromatography. pHEMA
170 and pNIPAM were analyzed in DMF + 0.1 % LiBr (w:v) and pure THF (Biosolve) respectively. 600 µl of
171 the samples were freeze dried and resuspended in 300 µl of the corresponding analytical solvent.
172 Polymer suspensions were centrifuged 10 min at 3000 rpm to pellet the insoluble materials. Size
173 exclusion separations were performed on a HPLC–UV/RI/MALS coupling consisting of a LC U3000
174 system (DIONEX) connected to a refractive index (RI) detector RI-101 (Shodex) or a DAWN 8+ MALLS
175 detector (Wyatt) UV. RI data were processed with the Chromeleon Data System (DIONEX) and MALLS
176 data with ASTRA software (Wyatt). Sample extracts were separated on a 2 PLgel Mixed-C column
177 (300x 7.5 mm, 5 µm, Agilent) thermostated at 30 °C. Flow rate was 1 ml/min and separation was
178 performed during 30 min. For RI detection, calibration was performed by poly(methyl methacrylate)
179 standard (Sigma-Aldrich).

180 3. Results

3.1. Selection and identification of phylloplane bacterial isolates

190 phylloplane bacterial strains were isolated from four crops: 58 from sugar beet, 51 from cabbage, 42 from rapeseed and 39 from corn. Four polyacrylic water-soluble linear polymers (pHEMA, pNIPAM, pAA and pAM) and a hydrophobic triglyceride-based monomer (ESOA) were used as selective substrates to obtain bacterial strains able to grow on long chains compounds with low susceptibility to enzymatic degradation or with low bioavailability. After 7 days of incubation with one of these substrates as sole carbon source, absorbance variations were recorded and used as a first selective criterion.

98 isolates were selected, because they triggered positive $\Delta OD_{600\text{ nm}}$ with at least one substrate. Considering all selective substrates, proportions of isolates per plant displaying a significant positive $\Delta OD_{600\text{ nm}}$ ranged from 10 to 22 % of the total isolates collection. Selection on ESOA allowed to obtain the highest number of strains (44 isolates), and selection on pNIPAM the lowest (20 isolates). Cabbage, rapeseed and sugar beet were the main sources of isolates growing with ESOA. About 15 % of the bacterial bank showed a positive $\Delta OD_{600\text{ nm}}$ with pHEMA, pAS and pAM, with isolates from the 4 plant species. pHEMA and pAS revealed similar profiles with percentages of isolates from cabbage and sugar beet around 7 % each, and from corn and rapeseed around 2 %. pAM showed a more equilibrated profile between origins with close percentages of isolates from sugar beet, rapeseed and corn (between 2.6 and 4.2 %) and a slightly higher percentage of isolates from cabbage (7 %). Isolates from sugar beet and cabbage were overrepresented regarding growth results with pNIPAM (around 5 % each). Regarding all substrates, sugar beet and cabbage were the most represented degradative strains providers.

17 isolates out of 98 were further selected on the basis of their versatility towards the substrates (absorbance increase for the same strain with different xenobiotics) or the intensity of the growth (highest $\Delta OD_{600\text{ nm}}$ with at least one substrate). In order to prevent the study of artifacts or of non-reproducible growths, $OD_{600\text{ nm}}$ kinetics of the seventeen selected isolates with the substrate(s) suspected to be degraded were checked to fit with classical bacterial growth curves before further characterizations. These isolates were identified through sequencing of their 16S rDNA and belong to 3 phyla (Firmicutes, Actinobacteria and Proteobacteria) covering 6 families (Bacillales Family XII. Incertae Sedis, Bacillaceae, Nocardiaceae, Micrococcaceae, Enterobacteriaceae and Pseudomonadaceae).

3.2. Growth of the selected strains according to the nature of the selective substrate

A principal component analysis (PCA) was performed with the 17 selected strains as an attempt to identify key parameters driving their capacity to grow with the selective substrates (Figure 1). This data analysis was done on $\Delta OD_{600\text{ nm}}$ obtained after 7 days of incubation with all selective substrates.

The PCA indicates a correlation between pHEMA and pNIPAM through the bacterial strains able to grow with them as sole carbon source. Both substrates present side chains on their hydrocarbon backbone, which could be more readily cleaved and used as carbon source. ESOA and pAM appear also correlated, although they do not share any chemical or physical characteristics. The angle formed between pAS and other variables suggests that the values recorded for this substrate are rather independent.

The PCA allowed to distinguish main groups corresponding to the family branches of the phylogenetic classification. This observation suggests that the degradative capacities for the different tested substrates are rather driven by the phylogeny of the bacteria than by their plant of origin. *Exiguobacterium sibiricum* strains, which belongs to the Bacillales Family XII. Incertae Sedis, formed an ellipse corresponding to higher $\Delta OD_{600\text{ nm}}$ with pHEMA and pNIPAM as sole carbon source. Micrococcaceae strains generally showed positive $\Delta OD_{600\text{ nm}}$ with at least 3 substrates including ESOA and pHEMA, which explains their central position. While being a water-soluble polymer, pHEMA also possesses a non-polar methyl group. Thus, changes in the cell membrane hydrophobicity or production of biosurfactants might be common mechanisms to access pHEMA and ESOA in these strains. Nocardiaceae contained two *Rhodococcus* species, whose growths were more favored on

232 pAM and ESOA, though *R. fascians* showed also a positive $\Delta OD_{600\text{ nm}}$ with pHEMA and appears more
233 central.

234 **3.3. Growth profiles analyses**

235 Growth kinetics were recorded to further characterize growth parameters and in particular
236 maximal growth and exponential growth rate (Figure 2).

237 The growth using ESOA as unique carbon source was confirmed for 10 out of the 17 bacterial
238 strains. For half of them, growths occurred only with ESOA. Four strains (*Exiguobacterium sibiricum*
239 2, *Arthrobacter koreensis*, *Rhodococcus fascians* and *Erwinia persicina*) could grow with both ESOA
240 and pHEMA. *Micrococcus luteus* displayed growth kinetics with ESOA and pNIPAM. The maximal
241 growths were generally higher with ESOA than for the other substrates, which could be in relation
242 with a more efficient use of this molecule. Exponential growth rates observed with ESOA were also
243 higher in average and did not rely upon the maximal absorbance. This could imply diversity in the
244 metabolic pathways enabling the use of this substrate or in the efficiency to have access to it. All the
245 bacterial families were represented among ESOA degraders underlying the fact that oily substrates
246 may be more commonly catabolized. *Bacillus* species were overrepresented (40 %), which is in
247 accordance with the literature widely describing this genus for oily substrate degradation. *Bacillus sp.*
248 3 showed the highest maximal growth, but had the lowest growth rate. This may imply a low
249 efficiency in the use of ESOA or a limited access rate to it. On the contrary *Bacillus thuringiensis* and
250 *Bacillus sp.* 1 exhibited both high maximal growths and μ values, indicating a good access to the
251 substrate and a high catabolic activity. The highest growth rate was recorded for *E. sibiricum* 2, which
252 was the only *E. sibiricum* strain able to grow with ESOA.

253 pHEMA led to 10 reliable growth kinetics over the 17 selected strains. 60 % of the strains
254 were identified as *E. sibiricum*. Among them, four grew on pHEMA only, five also with pNIPAM and
255 one also with ESOA. *E. sibiricum* 1 and 6 exhibited the highest maximal growth together with the
256 highest exponential growth rate. The other *E. sibiricum* strains showed lower growth capacities,
257 underlying an intraspecies diversity.

258 5 strains could grow with pNIPAM as unique source of carbon. The variations of their
259 maximal growth and their exponential growth rate were well correlated for this substrate. As for
260 pHEMA, the species *E. sibiricum* was well represented (3 out of 5 pNIPAM degraders). *E. sibiricum* 1
261 exhibited the highest maximal growth as well as the highest exponential growth rate for pNIPAM and
262 pHEMA. *E. sibiricum* 3 and 6 as well as *P. nitroguajacolicus* could also use both pNIPAM and pHEMA.
263 Only *M. luteus* grew with pNIPAM and ESOA.

264 **3.4. Substrates chemical alterations by promising isolates**

265 *Bacillus sp.* 1, *E. sibiricum* 1 and *A. koreensis* were selected to study the alteration of ESOA,
266 pNIPAM and pHEMA, respectively, because of their high maximal growth coupled with a high
267 exponential growth rate. The characterization of the chemical alterations was conducted through
268 culture supernatant analyses using GPC, ^1H NMR and FTIR.

269 **3.4.1 ESOA alterations by *Bacillus sp.* 1**

270 The lack of growth in MM without any carbon source confirms that the observed growth with
271 ESOA is due to the use of this molecule as carbon source. In contrast to the control with glucose
272 exhibiting a lag phase, growth with ESOA starts immediately (Figure 3a). This phenomenon could rely
273 either on a higher specialization toward hydrophobic substrates through hydrophobic cell surfaces or
274 on the production of biosurfactants. ^1H NMR analyses were carried out on the supernatants of both
275 abiotic control and bacteria-inoculated culture medium to detect the modification of ESOA and/or
276 the production of new compounds. The abiotic control with ESOA but without bacteria also enabled
277 to check that, despite its hydrophobic nature, ESOA did not totally sediment through centrifugation.
278 Indeed, ^1H NMR ESOA characteristic signals could be observed in the control supernatant at 0.9-1.5
279 ppm (CH_2 and CH_3 , fatty chain), 2.3 ppm ($\text{CH}_2\text{-C=O}$), 4.1-4.3 (CH_2 , glycerol group), 3.5-5.5 ppm ($\text{CH}_2\text{-O}$,
280 fatty chain), 5.25 ppm (CH , glycerol group), 5.9-6.4 ppm (acrylic group). The ESOA content in the
281 culture supernatants was estimated by the $\text{CH}_2\text{-C=O}$ signal intensity as compared to a standard
282 (pyridine) added in the sample. An oil depletion of 83.1 % was recorded in the culture supernatant
283 after incubation. This correlates well with the high maximal growth observed, in good agreement

284 with the hypothesis of a biodegradation of ESOA by *Bacillus sp. 1*. Acrylate groups could not be
285 observed anymore on the ^1H NMR spectra after incubation of ESOA with the bacteria and CH_2 peaks
286 of glycerol group dramatically decreased, excluding only adsorption phenomena. These results rather
287 suggest a hydrolysis of the ESOA ester functions and consequently the production of esterase by the
288 bacteria. Besides, a higher ratio of CH_2 and CH_3 groups to $\text{CH}_2\text{-C=O}$ group was observed in the culture
289 media spectra, which could not only be attributed to ESOA degradation, but may rely on the
290 production of bacterial extracellular molecules such as biosurfactants (Figure 3b).

291 The FTIR spectrum of pure ESOA (Figure 3c) shows bands at 2900, 1724, 1627 and 1186 cm^{-1}
292 due to CH stretching, C=O stretching of ester groups, C=C stretching of acrylic groups and C-O-C
293 stretching of ester functions, respectively. The C=C stretching of acrylic groups (1627 cm^{-1}) and C-O-C
294 stretching of ester functions (1186 cm^{-1}) was no more observed after incubation with *Bacillus sp. 1*
295 (Figure 3c). This result is in accordance with NMR data showing a decrease of the oil content and the
296 disappearance of glycerol and acrylic groups in the presence of the strain. The hypothesis of ESOA
297 hydrolysis by esterases could be confirmed by the presence of signals around 1750 cm^{-1} that can be
298 attributed to a COOH group.

299 3.4.2 pNIPAM alterations by *Exiguobacterium sibiricum 1*

300 *E. sibiricum 1* showed the most noteworthy growth kinetics with pNIPAM in term of both
301 maximal absorbance and growth rate (Figure 4a). No growth was detected in MM, confirming that
302 growth observed in other culture conditions relied on the presence of a carbon source only. *E.*
303 *sibiricum 1* could grow with pNIPAM over the 48 first hours, whereas growth decreased after 27
304 hours in the positive control containing glucose. The reason of this phenomenon remains to be
305 investigated, but the absence of stationary phase could reflect the production of a toxic metabolite
306 under this culture condition. GPC analyses were performed on freeze-dried culture supernatant in
307 THF in order to evaluate polymer molecular weight modifications. Native pNIPAM has a molecular
308 weight close to 1200 g/mol as determined by Refractive Index (RI) measurements using pMMA as
309 standard. Size exclusion chromatography (SEC) chromatograms of pNIPAM after 4 days of incubation
310 with *E. sibiricum 1* showed a major peak at 17.4 min, which corresponds to a 470 g/mol molecular
311 weight (Figure 4b). The shift of pNIPAM signal is compatible with an alteration of the polymer such as
312 backbone modification or an amide bond hydrolysis.

313 ^1H NMR was performed on the culture supernatant to confirm a possible alteration of the
314 polymer (Figure 4c). ^1H NMR profile of culture supernatants after incubation with *E. sibiricum 1* is
315 very close to the abiotic control. The characteristic peaks of pNIPAM can be observed at 3.76 ppm,
316 1.94 ppm, 1.5 ppm, 1 ppm corresponding to -C-H of the isopropylamine group, -C-H in position alpha
317 of the amide group, -C=O and - CH_2 groups of the carbon backbone, - CH_3 group of the isopropylamine,
318 respectively. The signals at 2.6 ppm and 2.3 ppm were attributed to the CH_2 groups of the terminal
319 function ($\text{C}_3\text{H}_5\text{O}_2\text{S}$). Terminal function of pNIPAM can be used as an internal standard to estimate the
320 relative number of monomer units (noted "n"). ^1H NMR underlined a 18.3 ratio of the polymer
321 backbone - CH_2 - as compared to the terminal functions ($\text{C}_3\text{H}_5\text{O}_2\text{S}$). The n value dropped to 17.2 in the
322 presence of *E. sibiricum 1*. So, the main backbone of pNIPAM seems to be degraded in smaller linear
323 polymeric units. The same method was used to determine the isopropylamine unit number and the
324 ratio between the monomer and isopropylamine unit numbers. A small decrease in this ratio was
325 recorded in the presence of *E. sibiricum 1*. This observation suggests the partial hydrolysis of amide
326 bonds, thus liberating isopropylamine in the culture medium, which may be used as a carbon source
327 for bacterial growth. The low modifications detected by ^1H NMR spectra fits well with the low growth
328 of the bacteria observed by $\text{OD}_{600\text{nm}}$ monitoring.

329 The culture supernatant was also analyzed by the FTIR technique and compared to the native
330 polymer (Figure 4d). Characteristic pNIPAM bands could be recorded in *E. sibiricum 1* culture
331 supernatants at 1628, 1558, 1460 and 1390 cm^{-1} , corresponding to amide bands I, II and III,
332 respectively. This observation is in accordance with ^1H NMR spectra, which confirmed the presence
333 of remaining pNIPAM in the culture supernatants. New bands around 1700 cm^{-1} and at 1080 cm^{-1} in
334 *E. sibiricum 1* culture supernatant suggests the appearance of carbonyl groups (C=O stretching) and

335 C-O or C-N groups of alcohols, lactones or amines. They may be related to polymer oxidation or to
336 the production of new bacterial metabolites.

337 **3.4.3 pHEMA alterations by *Arthrobacter koreensis***

338 The isolate identified as *A. koreensis* was selected to study the pHEMA alteration (Figure 5).
339 No growth was observed in the negative MM control without substrate confirming that the growth
340 observed in other culture conditions was due to the presence of an organic carbon source. The
341 growth kinetics recorded with pHEMA was similar to the one obtained with glucose in the 10 first
342 hours (Figure 5a). No lag phase was observed, and an exponential growth phase started after 45 min
343 of incubation in both conditions with μ of 0.159 ± 0.005 and 0.139 ± 0.005 for glucose and pHEMA
344 respectively. This exponential growth stopped earlier with pHEMA, after 6h of incubation, compared
345 to 13h with glucose. The maximum OD_{600 nm} was 0.165 ± 0.015 at 10 hours for pHEMA and then
346 absorbance slightly decreased contrary to glucose for which maximal absorbance reached 0.348
347 ± 0.010 after 45h. This profile could indicate a limitation in usable substrates or an accumulation of
348 toxic metabolites with pHEMA.

349 A decrease in pHEMA molecular weight, from 21,800 g/mol in the abiotic control to 3150
350 g/mol in *A. koreensis* culture supernatant was determined by SEC analysis (Figure 5b).

351 No signal corresponding to native pHEMA could be recorded in *A. koreensis* culture
352 supernatants through ¹H NMR analysis (Figure 5c). The presence of new peaks in the region of 7-8
353 ppm (characteristic of aromatic compounds), and 1-2 ppm (CH₂, CH₃) suggest that new metabolites
354 related to the bacterial development did appear in the supernatants.

355 The FTIR spectrum (Figure 5d) obtained with the *A. koreensis* culture supernatant was very
356 similar to the pure pHEMA ones with the presence of the characteristic bands at 2940 cm⁻¹ and 2855
357 cm⁻¹ (CH), 1702 cm⁻¹ (C=O), 1452 cm⁻¹, 1365 cm⁻¹, 1154 cm⁻¹ (C-O-O-R), 1071 cm⁻¹ (C-OH).

358 **4. Discussion**

359 **4.1. Ecology and biodegradation activities of the isolated species**

360 Genera identified in this study are recurrently isolated from soil, water and both
361 phyllosphere and rhizospheres of crops and ornamental plants. *E. sibiricum* have been isolated from
362 different types of soils, including crop fields, permafrost or industrial contaminated soils, as well as
363 from aquatic environments including marine sediments or industrial water effluent (Vishnivetskaya
364 *et al.*, 2009). The isolates selection also includes four species of the *Bacillus* genus, among which one
365 clearly identified as *Bacillus thuringiensis*. The 3 other strains could not be determined at the species
366 level, but presented a 16S rRNA sequence similarity of 98 % with *B. pumilus*, *B. weihenstephanensis*,
367 *B. simplex*, *B. cereus*, *B. licheniformis* and *B. subtilis*. All those strains have been previously isolated
368 from very different types of soils such as cotton fields, lakeshores, mangrove (Anwar *et al.*, 2009;
369 Lima de França *et al.*, 2015) and oil contaminated soils (Al-Sharidah *et al.*, 2000; Bezza and Chirwa,
370 2015; Calvo *et al.*, 2004; Maiti *et al.*, 2013; Kebria *et al.*, 2009). *B. pumilus* and *B. licheniformis* were
371 also isolated from plant rhizospheres (Gutiérrez-Mañero *et al.*, 2001). The *Bacillus* strains identified
372 in this study were recovered from all the sampled plants, underlying the presence of this genus in a
373 diversity of habitats within plant phyllospheres. Rhodococci were also described in seawater,
374 sediments, polluted soils, plant rhizosphere and plant surfaces (Larkin *et al.*, 2006). *Arthrobacter* is
375 representative of soil bacteria (Lee *et al.*, 2003). Among the genus *Erwinia*, *E. persicina* is a
376 phytopathogenic strain with a broad host range (Zhang and Nan, 2014), and was found in the
377 phyllosphere of rape in this study. The presence of several strains of *Bacillus*, *Arthrobacter*,
378 *Pseudomonas* and *Micrococcus* with hydrocarbon degradation properties in the phyllosphere of a
379 crop panel was also reported previously (Al-Awadhi *et al.*, 2012; Ilori *et al.*, 2006).

380 Degradation activities for recalcitrant and hydrophobic polymers have already been reported
381 for some of the identified genera. *Exiguobacterium sp.* has been identified as a polystyrene (PS)-
382 degrading microorganism present in the gut of mealworms, the larvae of *Tenebrio molitor* Linnaeus
383 (Yang *et al.*, 2015). Besides, compounds entering the composition or used for the synthesis of plastic
384 materials can be catabolized by *Arthrobacter* species. *Arthrobacter keyseri* was shown to catabolize
385 phthalate *via* a plasmid-encoded pathway (Eaton, 2001). *Arthrobacter sp.* P1 could use methylamine
386 as sole carbon source and degrade it *via* amine oxidase and facultative methylotrophic metabolisms

387 (Levering *et al.*, 1981). In addition, all the genera obtained through our screening were reported to
388 possess degradative capacities for other types of recalcitrant molecules. This includes pesticides,
389 alcohols, heterocyclic compounds, halogenated, sulfonated compounds and steroids (Larkin *et al.*,
390 2006; Tallur *et al.*, 2008; Laczi *et al.*, 2015; Kasana and Pandey, 2018) as well as oil components
391 (Wright *et al.* 1993; Seo *et al.*, 2006; Kumar *et al.*, 2008; Mohanty and Mukherji, 2008; Binazadeh *et*
392 *al.*, 2009; Bezza and Chirwa, 2015). However, none of these previous works has associated the
393 identified species with the degradation of the substrates used in our study.

394 A critical aspect in hydrophobic compounds degradation such as ESOA resides in the access
395 to the substrates. Interestingly, several of the identified genera were previously described either to
396 be able to modify their cell surface hydrophobicity or to produce tensioactive molecules. The cell
397 surface hydrophobicity modification was reported for *Exiguobacterium* (Mohanty, 2006; Mohanty
398 and Mukherji, 2008). The production of tensioactive molecules was described for *Exiguobacterium*,
399 *Bacillus* (Calvo *et al.*, 2004; Thavasi *et al.*, 2008; Cai *et al.*, 2017), *Rhodococcus* (Bell *et al.*, 1998),
400 *Arthrobacter* (Morikawa *et al.*, 1993), *M. luteus* (Tuleva *et al.*, 2009) or *Pseudomonas* (Kumar *et al.*,
401 2008; Noordman *et al.*, 2002).

402 The identification of our isolates is thus in great accordance with the largely furnished
403 literature concerning polymers or hydrophobic molecules biodegradation, which tends to validate
404 our isolation and screening strategy to target bacteria able to use this kind of molecules as
405 substrates.

406 **4.2. Relations between growth with selective substrates and phylogeny of the isolates**

407 The analysis of $\Delta OD_{600\text{ nm}}$ results in light of isolate identifications enabled to underline the
408 selection of certain bacterial families. Several strains belonging to the *Rhodococcus* genus were
409 shown to grow with pAM as substrate in our study. Bacteria of this genus were recurrently found in
410 soils and could thus have already been exposed to pAM, which is used in agriculture as water
411 absorbent to maintain soil humidity (Wenhua *et al.*, 2008). Bacillaceae were generally selected for
412 positive $\Delta OD_{600\text{ nm}}$ with ESOA and were less versatile. As mentioned previously, *Bacillus* can be found
413 in diverse environments and have been described to be associated with the degradation process of a
414 wide range of alkanes and hydrophobic compounds (Al-Sharidah *et al.*, 2000; Bezza and Chirwa;
415 2015, Calvo *et al.*, 2004; Maiti *et al.*, 2013; Kebria *et al.*, 2009). This suggests that species of this
416 genus may possess specialized catabolic pathways to degrade waxes from the phyllosphere (Kunst
417 and Samuels, 2003; Buschhaus *et al.*, 2007; Buschhaus and Jetter, 2012). Enterobacteriaceae and
418 Pseudomonadaceae only contained one strain each and were more associated with ESOA, but these
419 strains exhibited low $\Delta OD_{600\text{ nm}}$ in the screening.

420 When considering growth capacities of isolates in view of stem size of the plant of origin, our
421 results tend to show that bacteria from the phyllosphere of more aerial plants are more efficient in
422 oily substrate utilization. This was mainly observed for the species from the *Bacillus* genus that
423 appeared to preferentially utilize the triglyceride derivative ESOA. However most of the strains of
424 interest were obtained from cabbage or sugar beet. Both of these plants are short-stem crops
425 growing close to the soil surface, which is likely to enhance the isolation of soil-borne bacteria
426 described as one of the main phylloplane microbial communities (Pieterse *et al.*, 2016). Notably
427 *Exiguobacterium* strains were isolated from leaves of cabbage and sugar beet, and the *Arthrobacter*
428 strain and the two *Rhodococci* came from sugar beet. These genera were isolated from soil in other
429 studies as previously mentioned. These isolates appeared to be more polyvalent regarding
430 substrates, which is correlated with the abundant literature about the capacity of these species to
431 degrade a wide range of xenobiotics. Moreover, soils are environmental compartments with a high
432 diversity of substrates and of microorganisms, which could explain a greater catabolic diversity
433 resulting from the competition for substrates. Thus, in supplement to be a source of phylloplane
434 microorganisms adapted to wax degradation, phyllosphere could be regarded as an environment
435 naturally rich in hydrophobic and long chain compounds selecting strains of interest for xenobiotics
436 biodegradation notably from the soil biodiversity.

437 Analyses of growth kinetics parameters enable to complete these interpretations. All the
438 genera efficiently growing with ESOA as sole carbon source have been described to contain species

439 able to produce biosurfactants, notably lipopeptides for *Bacillus* species (Ben Ayed *et al.*, 2015; Bezza
440 and Chirwa, 2015; Fooladi *et al.*, 2016; Lima de França *et al.*, 2015; Parthipan *et al.*, 2017; Yuan *et al.*,
441 2011). *E. sibiricum* 2 was the only *E. sibiricum* strain able to grow with ESOA and displayed the
442 highest growth rate. Most of the strains growing with pHEMA are also identified as *E. sibiricum* in our
443 study. *Exiguobacterium* bacteria have already been described to exhibit a wide range of catabolic
444 activities such as the production of lipases and esterases (Vishnivetskaya *et al.*, 2009; Ali *et al.*, 2015;
445 Kasana and Pandey, 2018), which could explain our results with ESOA and pHEMA. All strains
446 selected for their growth ability with pNIPAM could grow with at least one other substrate, mainly
447 pHEMA with notably 3 strains of *E. sibiricum* able to grow with the two polymers.

448 Growth on pAM and pAS observed in the first screening step for some isolates through OD
449 variation measurements could not be reproduced for growth kinetics. The rare works reporting the
450 biodegradation of pAM by isolated strains describe a degradation process occurring on time scales up
451 to 14 days (Matsuoka *et al.*, 2002; Nakamiya and Kinoshita, 1995; Song *et al.*, 2017; Wen *et al.*,
452 2010). It may be possible that our growth kinetic studies over 4 days only may not be sufficient to
453 obtain a visible effect. Besides, the pAM used in our study could also have a too high molecular
454 weight (40,000 g/mol) limiting its biodegradation. Biodegradation generally decreases with an
455 increased polymer molecular weight (Hayashi *et al.*, 1993; Hayashi *et al.*, 1994). No degradation has
456 been reported for molecular weight over 4,000 g/mol and pAS used in our study was 5,000 g/mol.
457 Moreover, Nyssölä and Ahlgren (2019) recently reported that the pAS part would be more
458 recalcitrant to bacterial degradation than the amide part during pAM biodegradation and confirmed
459 that the biodegradation efficiency is notably linked to the polymer molecular weight.

460 **4.3. Chemical alterations associated with bacterial growth**

461 The chemical analyses confirm the isolation of a *Bacillus* *sp.* strain efficiently degrading ESOA.
462 This biodegradation appears to involve esterase activities in correlation with the literature reporting
463 esterases involvement in xenobiotics biodegradation by strains of this genus (Niazi *et al.* 2001; Shah
464 *et al.*, 2016). The precise metabolic pathways and generated metabolites have now to be further
465 investigated as well as the possibility of biosurfactants production suspected in our analyses.

466 Incubation of pNIPAM with *E. sibiricum* 1 led to a decrease of the polymer molecular weight,
467 the appearance of low molecular mass compounds, ¹H NMR signals alterations and the appearance
468 of C=O and C-O bands in IR spectra. These elements support the hypothesis of a polymer chain
469 oxidation that more presumably lead to a polymer alteration through the cleavage of the main
470 carbon chain. Such biodegradation potential of aliphatic and aromatic carbon structures by an
471 *Exiguobacterium* strain through an oxidative pathway is in accordance with the PS-degrading
472 capacities observed in this genus (Yang *et al.*, 2015). *E. sibiricum* could thus represent a strain of
473 interest for acrylic polymers bioremediation through these specific oxidative pathways. Indeed, *E.*
474 *sibiricum* strains isolated in our study also showed growth potential with pHEMA and triggered
475 polymer amount as well as polymer weight decreases in the culture supernatants, as determined by
476 SEC and ¹H NMR techniques. The appearance of new bands on the FTIR spectra is in accordance with
477 these results, suggesting pHEMA alterations resulting from bacterial activity.

478 To our knowledge, this study is the first clear observation that *Arthrobacter koreensis*
479 possesses a major degradation activity on a recalcitrant polymer. Mechanism of pHEMA alteration
480 could rely on the hydrolysis of the ethylene glycol lateral chain. Indeed the biodegradation process of
481 biopolymers such as polyhydroxyalcanoates (PHA), poly-β-hydroxybutyrate (PHB) and polylactic acid
482 (PLA) is based on the hydrolysis of ester or amide bonds (Emadian *et al.*, 2017). However pHEMA
483 alteration could also rely on the main long carbon chain oxidation with mechanisms closer to
484 hydrocarbon biodegradation (Abbasian *et al.*, 2015). Interestingly, the presence of genes dedicated
485 to the synthesis of plant growth promoters such as gibberellins, abscisic acid or strigolactones has
486 been reported in *A. koreensis* genome (Manzanera *et al.*, 2015). All these metabolites are deeply
487 related to molecules produced in isoprenoids or terpenoids metabolic pathways (Kuzma *et al.*, 1995;
488 Das *et al.*, 2007). The presence of metabolic pathways dedicated to the synthesis of long chain
489 isoprenoids could be a possible line of explanation for the pHEMA degradation abilities of this strain
490 either through reversible enzymatic reactions or through the direct use of partial degradation

491 products as precursors. In this respect, *A. koreensis* catabolic activities merit further investigations.
492 Its versatile metabolic activities make it a very promising species for biotechnological, environmental
493 and agricultural applications.

494 **5. Conclusions**

495 Plant phyllosphere was shown to be a suitable natural environment for the isolation of bacterial
496 strains with catabolic activities enabling functional polymers biodegradation. Resulting alterations
497 correspond to chemical structures oxidation and molecular weight decreases for ESOA, pNIPAM and
498 pHEMA.

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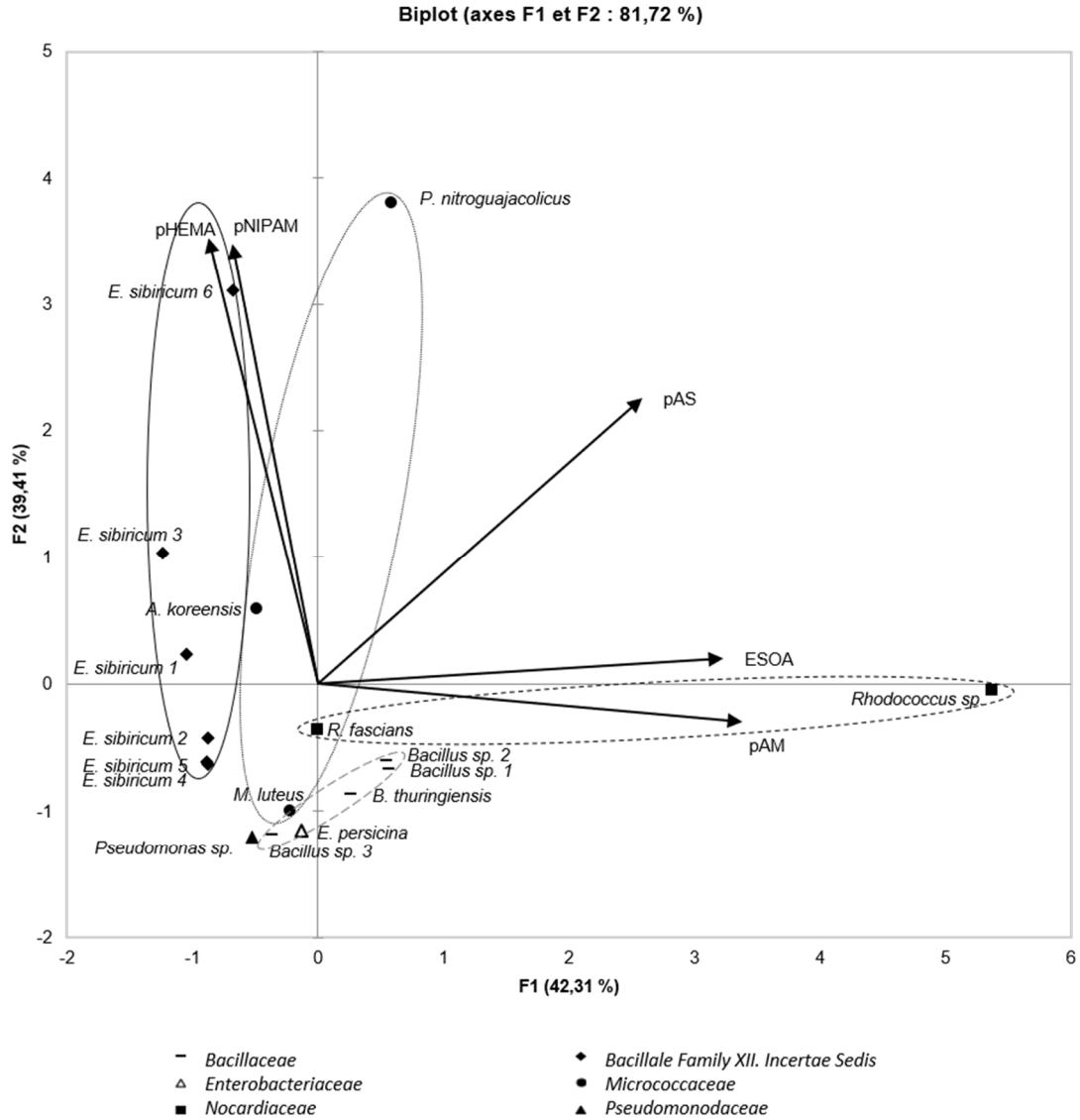
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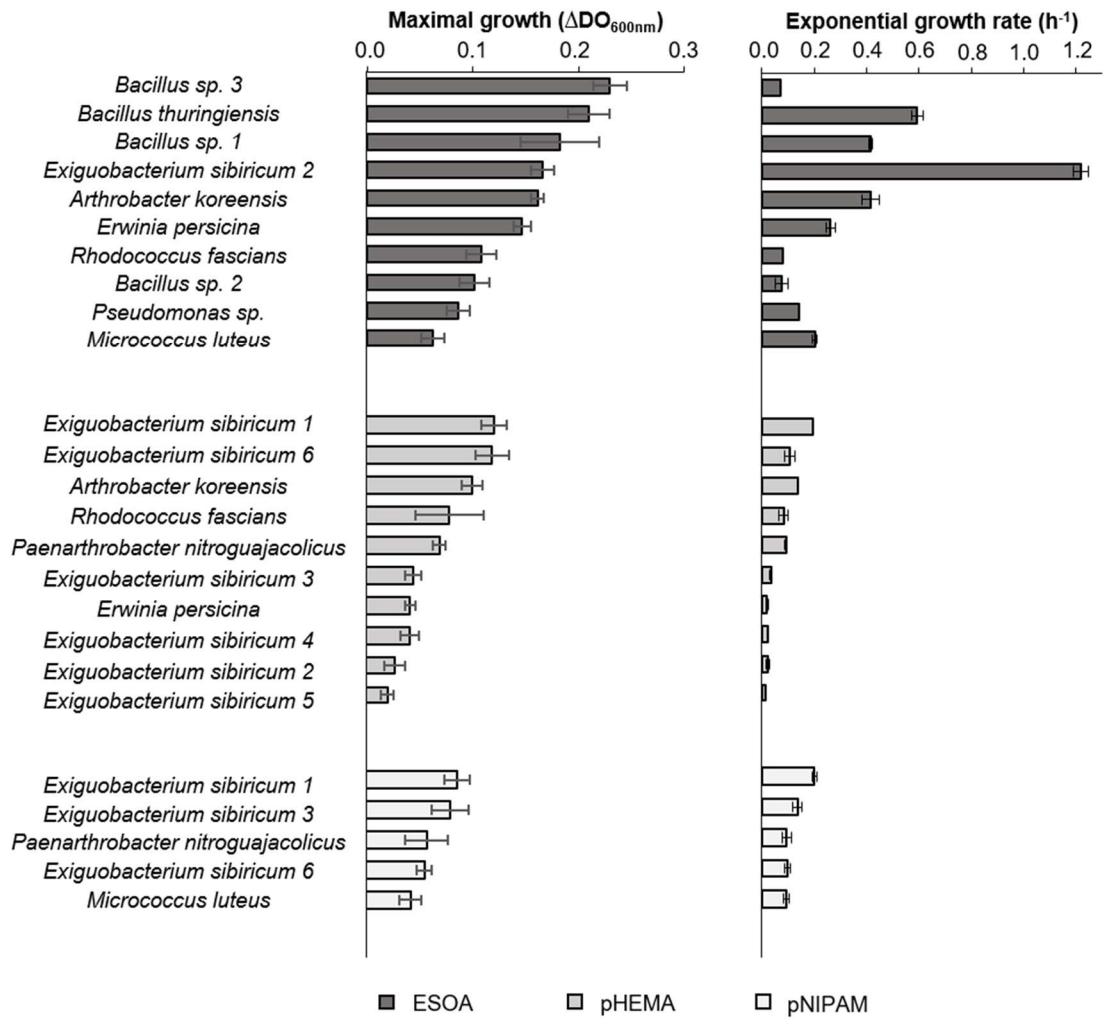
Table 1. Characteristics of selective substrates used in this study (information from the provider)

Substrate	Chemical structure	Mw g/mol	Concentration g/L
pAcrylamide		40,000	4
pAcrylate		5,100	5.84
pHEMA		20,000	3.6
pNIPAM		5,000	3.14
ESOA		990	2.6

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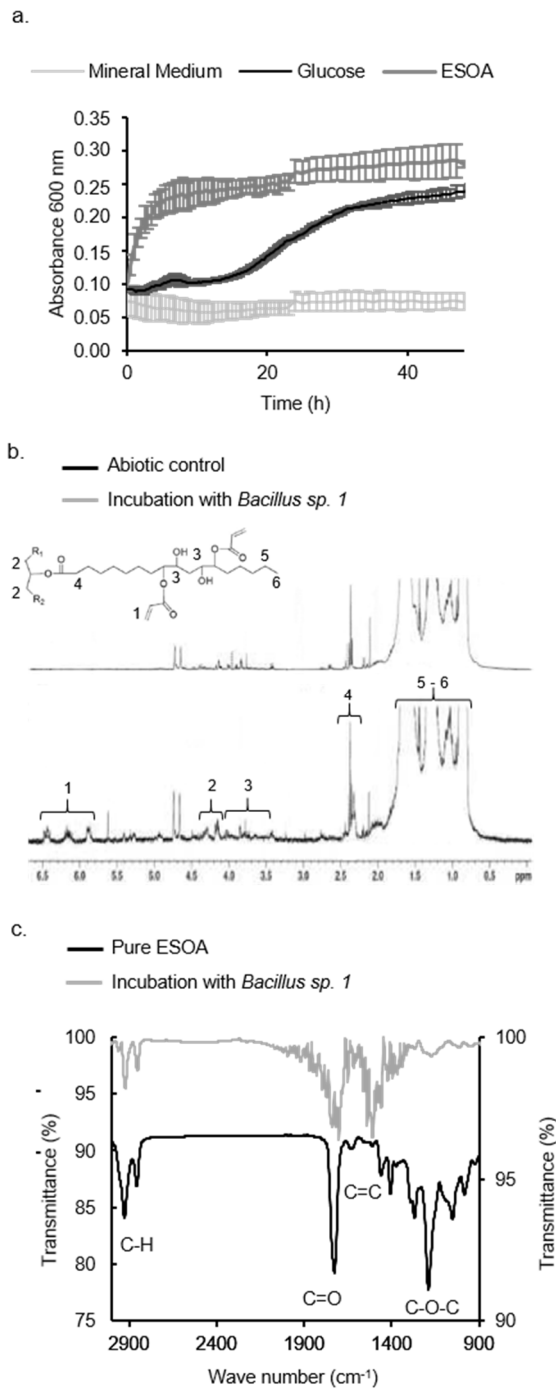


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 760 **Figure 1.** Principal Component Analysis (PCA) based on the ΔOD_{600nm} obtained after 7 days of
 761 incubation with each recalcitrant substrate for strains selected and identified after the qualitative
 762 screening. (81.7 % of the information is represented, ellipses underline pattern of bacterial families)



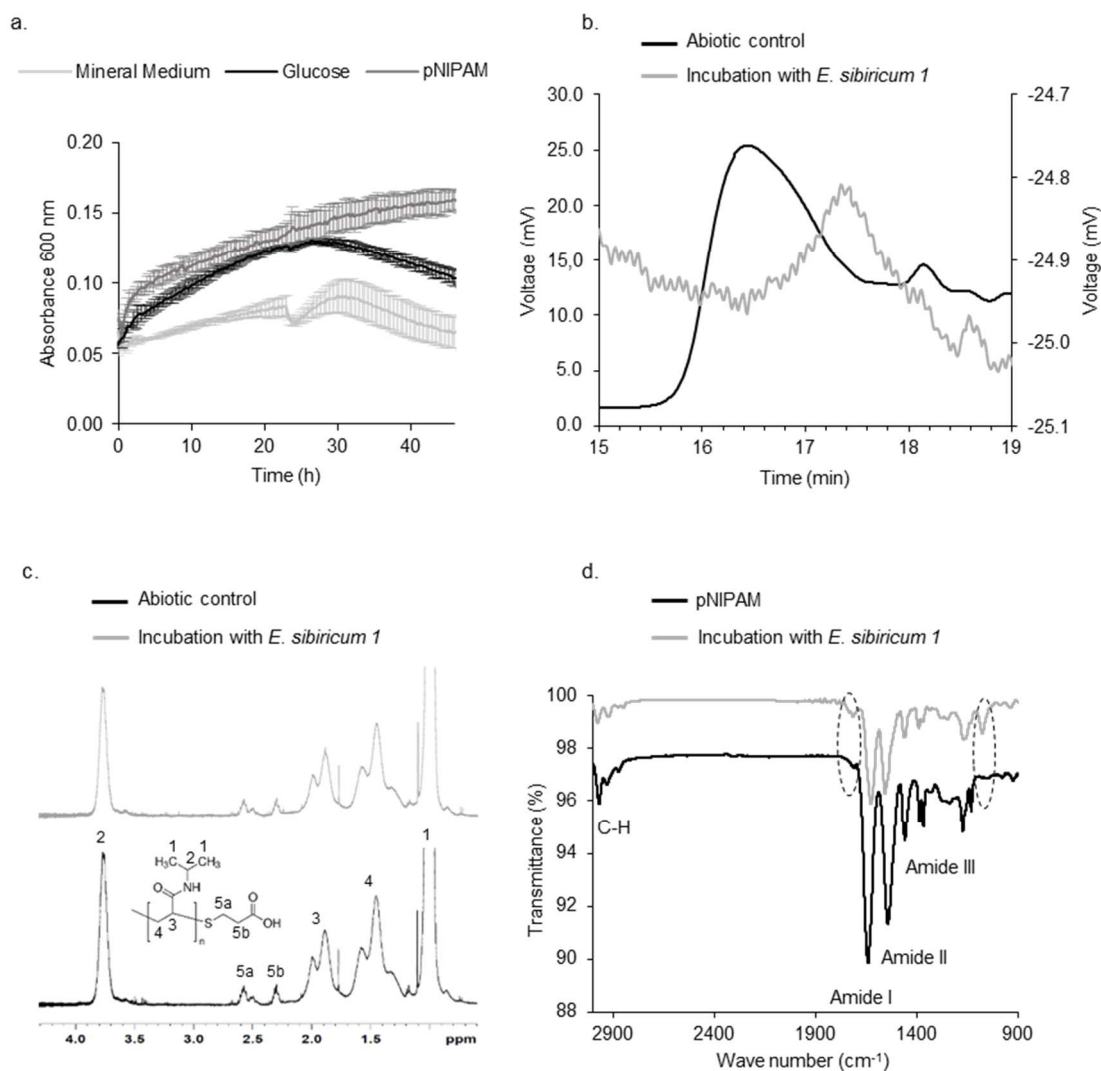
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Figure 2. Maximal growth and exponential growth rate obtained on 4 days of growth kinetics of crop leaves isolated bacteria with ESOA, pHEMA, and pNIPAM as sole carbon source.

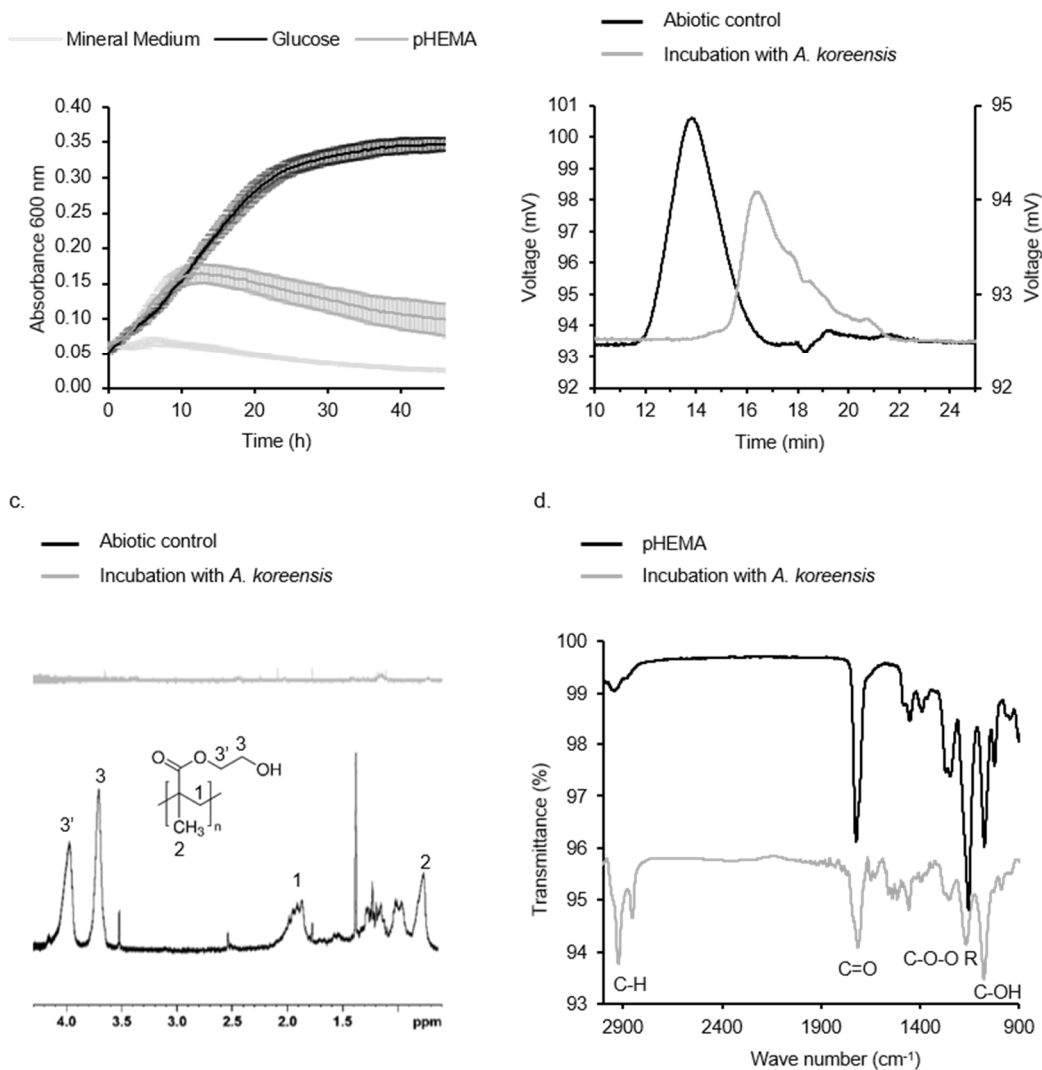


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Figure 3. Analysis of *Bacillus sp. 1* growth with ESOA as sole carbon source and characterization of alterations by comparing pure ESOA, abiotic control and the culture supernatants. a. Two first days of growth kinetic monitoring by 600 nm absorbance measurements, b. ¹H NMR spectra c. FTIR spectra



770
 771 **Figure 4.** Analysis of *Exiguobacterium sibiricum* 1 growth with pNIPAM as sole carbon source and
 772 characterization of polymer alterations by comparing pure pNIPAM or abiotic control with culture
 773 supernatants. a. Two first days of growth kinetic monitoring by 600 nm absorbance measurements,
 774 b. SEC analysis of culture supernatants using RI as detector, c. ¹H NMR spectra, d. FTIR spectra



775
 776 **Figure 5.** Analysis of *Arthrobacter koreensis* growth with pHEMA as sole carbon source and
 777 characterization of polymer alterations by comparing pure pHEMA or abiotic control with culture
 778 supernatants. a. Two first days of growth kinetic monitoring by 600 nm absorbance measurements.
 779 b. SEC analysis of culture supernatants using MALS as detector. c. ^1H NMR spectra d. FTIR spectra